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REPORTS

A SIMPLE AND SENSITIVE TEST FOR THE DETERMINATION OF PHENOLIC COMPOUNDS IN URINE AND ITS APPLICATION TO MELANOMA

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A simple procedure is outlined for the determination of phenolic compounds in urine. The method involves the diazotization of *p*-nitroaniline and subsequent coupling of the diazonium chloride with phenolic compounds. The diazo compound formed is determined by measuring the absorbance at 400 nm. The extinction coefficients for several compounds have been determined.

Application of this method to determine the levels of phenolic compounds in urine in patients with melanoma showed that the assay may be of diagnostic value. The amounts of phenols in 24-hr urine samples from patients with melanoma, patients with various other diseases, and healthy individuals were determined. A statistical analysis of these results showed that the results for patients with melanoma were higher than those for healthy individuals and patients with other diseases at 1% probability level. A comparison of this test with three other methods (Thormählen test, ferric chloride test, and ferrocitrate test) for determination of melanogens showed that the diazo test is the most sensitive one for detecting melanoma.

The excretion of melanin and melanin precursors (melanogens) has been employed as a possible diagnostic test for melanoma [1–18]. Two types of melanogens are known to be present in urine: phenol melanogens and indole melanogens [7]. The phenol melanogens are the early intermediate compounds and their metabolites in the series of reactions involved in the synthesis of melanin from tyrosine and dopa. The indole melanogens are formed later in the pathway leading to the synthesis of melanin. The melanogens have been determined by using colorimetric methods and paper chromatography. Two kinds of colorimetric methods have been mainly used for the determination of melanogens: (1) oxidation reactions in which the development of melanin or other reaction products are followed, and (2) the Thormählen test in which nitroferricyanide is added to urine. The nitrogen-free melanogens or phenol melanogens give only a positive oxidation test whereas the nitrogen melanogens or indole melanogens give positive reactions in both tests [7].

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Several compounds present in the urine samples from melanoma patients have been separated and characterized. There are more than 40 phenol melanogens such as *p*-hydroxyphenylacetic acid, salicyluric acid, homovanillic acid, *p*-hydroxyphenyl propionic acid, salicylic acid, ferulic acid, etc. [1]. The indole melanogens have been found to be mainly derivatives of 5,6-dihydroxyindole (Thormählen-positive indole melanogens) [16, 17] and of 5,6-dihydroxyindole-2-carboxylic acid (Thormählen-negative indole melanogens) [4].

In this communication we report a colorimetric method for the determination of phenolic compounds in urine. The method involves the reaction of an aromatic diazonium salt with phenolic compounds to yield colored diazo compounds [19]. Weiss has reported a qualitative method for the determination of urinary melanogens using the diazo reaction [20]. In our studies the diazo compound formed was quantitatively assayed spectrophotometrically. The reaction has been confirmed with several phenolic compounds as described below. Since a number of melanogens are phenolic compounds, the possibility of using this test for the diagnosis of melanoma was explored.

MATERIALS AND METHODS

p-Nitroaniline and sodium nitrite were obtained from British Drug Houses Ltd. L-Tyrosine and dopamine were purchased from Fisher Scientific Company and Mann Research Laboratory, respectively. Salicylic acid, DL-*p*-hydroxyphenyllactic acid, L-3,4-dihydroxyphenylalanine (dopa), biliverdin dihydrochloride, bilirubin, epinephrine, norepinephrine (L-arterenol bitartrate), 5-hydroxy

indole, 5-hydroxy indole-3-acetic acid, and indole-2-carboxylic acid were purchased from Sigma Chemical Company.

Twenty-four-hour urine samples were collected in bottles containing 10 ml of glacial acetic acid, 1 gm sodium sulfite, and 1 gm sodium oxalate as recommended by Hinterberger et al [8]. None of the patients or controls had taken salicylates during the 24 hr preceeding and during the collection period. All other drugs administered to the patients were noted. No attempt was made to regulate or note the diet of the subjects. Because of the limited number of cases available, the results have not been matched according to age or sex.

Standard Procedure for the Determination of Phenolic Compounds

One milliliter of 20% $NaNo_2$ is mixed with 10 ml of 0.5% *p*-nitroaniline in 1.0 N HCl. One-tenth milliliter of this mixture is added to 0.1 ml of the phenolic compound (or urine), and 3.8 ml of 0.5 M PO_4 buffer, pH 7.5, is added, and the contents are immediately mixed. The absorbance at 400 nm is determined after 30 min. The urine samples were appropriately diluted such that the absorbance values were proportional to the volume of the urine employed. The amounts of phenol melanogens in urine are expressed in terms of mg equivalent of tyrosine, calculated using the standard curve for tyrosine (Tab. I). In the early part of the study dopa was employed as standard. However, due to the unstability of this compound in solution, it was replaced by tyrosine for routine assays. Similar results were observed whether tyrosine or dopa was employed as the standard.

RESULTS

Using urine samples from healthy individuals with no known disease, the effects of various conditions of the reaction upon the absorbance were studied. Figure 1 shows the absorbance of the final product when various amounts of *p*-nitroaniline were employed. The absorbance of the reagent blank at 400 nm increased linearly with increasing amounts of *p*-nitroaniline. The absorbance of the sample tube steadily increased up to 0.5 mg of *p*-nitroaniline, and when the concentration of nitroaniline was further increased the absorbance increased at a slower rate. When the sample tubes were read against the reagent blank, the absorbance increased up to 1.0 mg nitroaniline and was not further increased when the amount of nitroaniline was further raised.

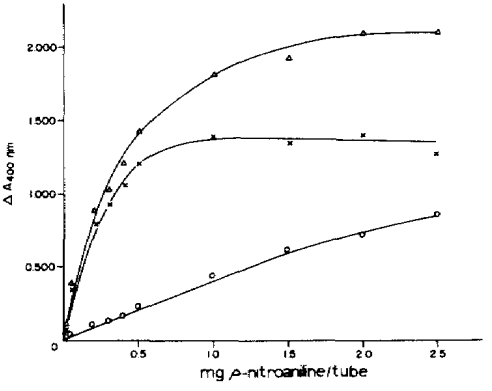

FIG. 1. Effect of varying the concentration of *p*-nitroaniline on the diazo test for phenolic compounds in urine. Δ——Δ = Absorbance of the sample tube read against water; O——O = absorbance of reagent blank; ×——× = absorbance of sample tube corrected for the absorbance of the reagent blank. Varying amounts of *p*-nitroaniline were added; other conditions were as in the standard procedure described under *Materials and Methods*. Urine containing melanogens equivalent to approximately 700 μg of tyrosine was added to each tube.

The dependence of the reaction on the concentration of $NaNO_2$ is shown in Figure 2. It may be seen that when the amount of $NaNO_2$ was less than 0.5 mg, the absorbance values for both the sample tube and the reagent blank were higher than 3.0. When the concentration of $NaNO_2$ was greater than 0.5 mg, the absorbance for the reagent blank was constant at approximately 0.2 and the absorbance values for the sample tube were also fairly constant. As a result, the absorbance of the sample tube read against the reagent increased rapidly up to 0.5 mg $NaNO_2$ and when the amount of $NaNO_2$ was further increased there was no significant difference in the absorbance. The dependence of the reaction on the final pH of the reaction mixture is shown in Figure 3. It was found that the absorbance of the sample tube increased rapidly as the pH was raised from 4 to 9.5. When the pH was further increased up to 12 the absorbance decreased. The absorbance of the reagent blank was very low up to pH 7.5 and then rapidly rose until pH 9.5 with a small decrease in absorbance between pH 10 and 12. The absorbance of the sample tube read against the reagent blank has a distinct optimum at 7.5. A small peak at pH approximately 9.75 was consistently observed.

Figure 4 shows the time course of the reaction and the stability of the reaction product as well as the absorbance of the diazonium reagent. The absorbance was read after varying periods of time following the preparation of the diazonium reagent and after mixing the reagents with the urine samples. The absorbance of the sample tubes rapidly increased up to 30 min after mixing, and after that the rate of increase was relatively slower. The absorbance of the reagent blank increased steadily up to 260 min. The absorbance of the sample tubes read against the reagent blank increased rapidly till 60 min, and thereafter the rate was relatively slower. The results of similar studies using several compounds are shown in Figure 5. All the absorbance values in this Figure have been corrected for the corresponding values for the reagent blank. With most of the compounds the absorbance increased rapidly for 15 to 20 min; there was only a very small increase after 30 min. On the contrary, with epinephrine and tyrosine the absorbance was found to increase for 90 min.

The absorption spectrum of the reaction product with normal urine is shown in Figure 6. There was a broad peak at 400 nm and the absorbance was considerably reduced above 500 nm. This spectrum is presumably the composite of the absorption

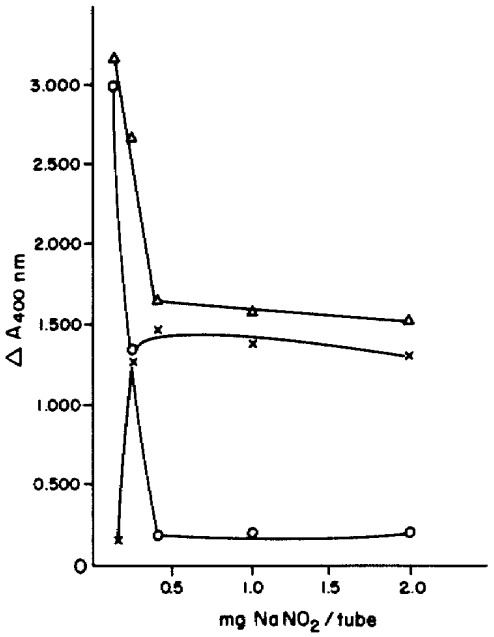

FIG. 2. Effect of varying the concentration of $NaNO_2$ on the diazo test for phenolic compounds in urine. Varying amounts of $NaNO_2$ were added. Other conditions were as in the standard procedure and the symbols as in Figure 1.

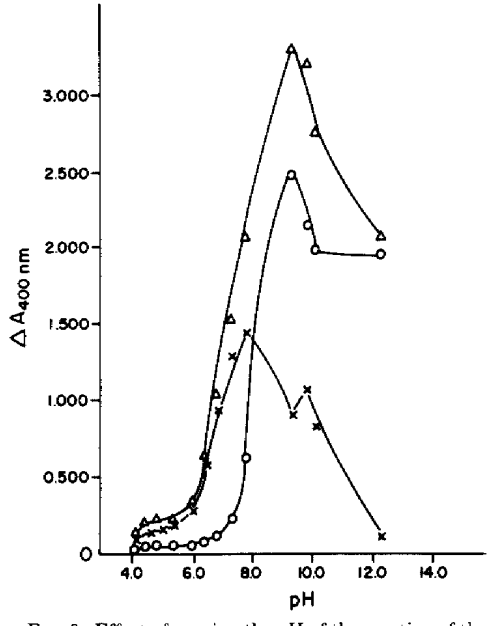

FIG. 3. Effect of varying the pH of the reaction of the diazo test for phenolic compounds in urine. The final pH was varied as shown in Figure 1. Other conditions were as in the standard procedure and the symbols as in Figure 1.

spectra of the reaction products of several compounds present in urine. Similar spectra were observed with several phenolic compounds also. Table I summarizes the results obtained with several phenolic compounds. The extinction coefficient for the reaction product for each compound was obtained by using a wide range of concentrations of the compound. The concentration range in which the absorbance was proportional to the concentration of the compound is also included in this Table.

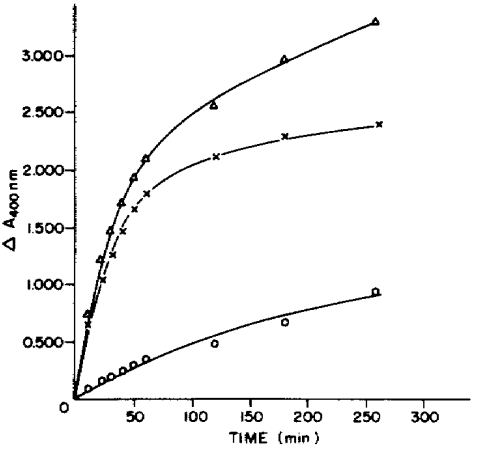

FIG. 4. Rate of formation of the reaction product with urine and the absorbance of the diazonium reagent. The conditions were as in the standard procedure. The amount of melanogens and the symbols were as in Figure 1.

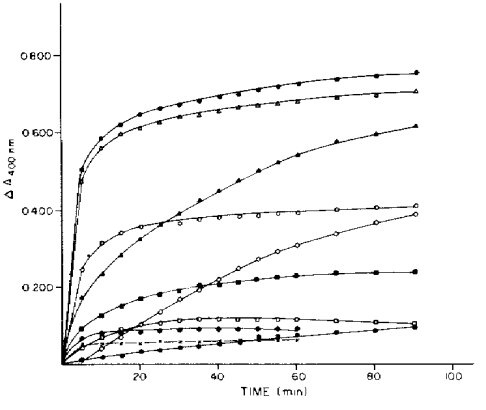

FIG. 5. Rates of formation of the reaction products with several phenolic compounds. ●——● = dopamine; △——△ = L-dopa; ▲——▲ = epinephrine; ○——○ = norepinephrine; ◇——◇ = L-tyrosine; ■——■ = 5-hydroxy indole; □——□ = DL-*p*-hydroxyphenyl acetic acid; ◆——◆ = bilirubin; ⊖——⊖ = salicylic acid; ×——× = biliverdin. One hundred micrograms of each compound were employed for the assay. The conditions for the assay were as in the standard procedure. All absorbance values have been corrected for the reagent blank.

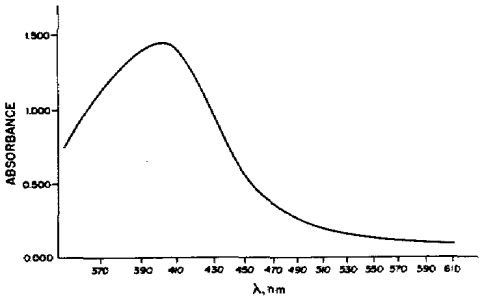

FIG. 6. Absorption spectrum of the reaction product with urine. The absorbance has been corrected for the reagent blank. The conditions were as in the standard procedure.

TABLE I. *Extinction coefficients for the reaction products from several phenolic compounds*

Various concentrations of the phenolic compounds were employed and the assay was carried out using the standard procedure described under *Materials and Methods*.

Compound	$E_{400\ nm}$	Concentration range (μm)
L-Dopa	7.58×10^4	0–10
Dopamine	9.07×10^4	0–10
L-Epinephrine	7.23×10^4	0–10
L-Arterenol bitartrate	7.18×10^4	0–10
L-Tyrosine	3.67×10^4	0–20
DL-*p*-Hydroxyphenyllactic acid	0.96×10^4	0–50
Bilirubin	2.87×10^4	0–50
Biliverdin dihydrochloride	1.50×10^4	0–100
Salicylic acid	1.16×10^4	0–100
Indole	4.18×10^3	0–20
5-Hydroxy-indole	9.07×10^3	0–20
5-Hydroxy-indole-3-acetic acid	2.57×10^4	0–20
2-Indole carboxylic acid	6.81×10^3	0–100

Samples of urine from patients with melanoma were examined by the above method; the standard procedure described above was employed for this purpose. Urine samples from healthy individuals and patients hospitalized with various diseases other than cancer were used as controls. Among the patients with melanoma, 8 had primary melanoma (2 uveal and 6 cutaneous). The results reported here were obtained at various periods following surgical removal of the tumor; at the time when the tests were performed all these patients were clinically free of tumor. The remaining 33 patients had either local recurrence or wide-spread metastasis. The results are expressed in terms of mg equivalents of tyrosine excreted during 24 hr, which was calculated using the standard curve for tyrosine (Tab. I). Figure 7 illustrates the mg equivalents of tyrosine in individual specimens of the above three groups. A statistical analysis of these results is given in Table II. The mean and standard deviation were calculated for each group. The results for the patients with melanoma were compared to those for healthy individuals and patients with other diseases. Similarly, the results for patients with diseases other than cancer were compared to those for the group of healthy individuals. Since the variances of the samples were different, Wallace's method was employed to calculate the degrees of freedom for Student's *t*-test [21, 22]. The results for the group of patients with melanoma were higher than those for healthy individuals or patients with other diseases at 1% probability level of significance. On the other hand, the results for the patients with diseases other than cancer were not significant even at 5% probability level when compared to healthy individuals.

The diazo test was employed to determine phenolic compounds in serum from the above patients. It was found that there was no significant difference among the values for healthy individuals, the patients with melanoma, and those with other diseases.

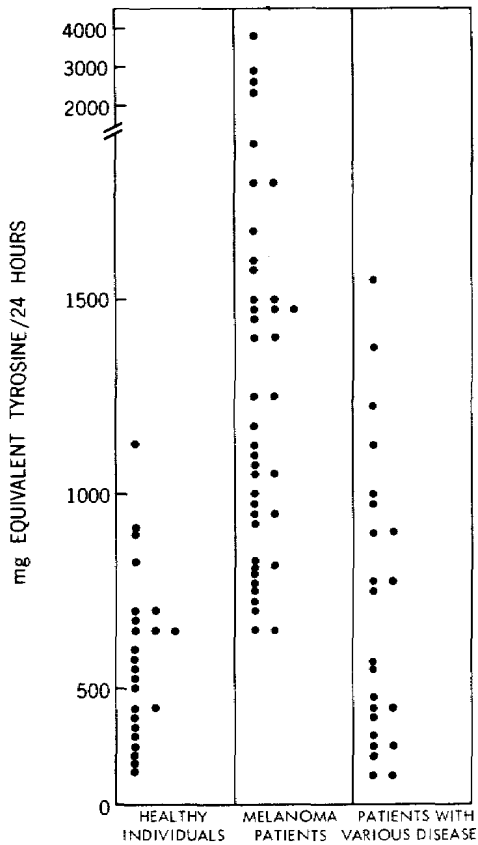

FIG. 7. Amounts of phenolic compounds in urine from healthy individuals, patients with melanoma, and patients with various other diseases.

TABLE II. *Comparison of phenolic compounds in urine from healthy individuals, patients with melanoma, and various other diseases*

Group	Mean	Standard deviation	Student's *t*-test
Healthy individuals	582	216	
Patients with melanoma	1342	647	
Patients with other diseases	743	378	
Patients with melanoma/Healthy individuals			6.85
Patients with melanoma/Patients with other diseases			4.87
Patients with other diseases/Healthy individuals			1.51

DISCUSSION

The above results describe the optimum conditions for the determination of phenolic compounds in urine using the diazotization reaction. The optimum conditions chosen for the test involve measuring the absorbance at 400 nm after 30 min following the mixing of all the reagents. It is seen from the results in Figure 4 that with urine the absorbance of the reaction product does not remain constant but continues to rise up to 260 min, although the rate of increase in the absorbance slows down considerably after 60 min. However, with several phenolic compounds tested, the absorbance does not increase considerably after 30 min. It would appear that one could choose any arbitrary interval in the region of 30 to 60 min for the measurements. There is a disadvantage in using the period of 60 min rather than 30 min; in tubes containing relatively higher amounts of phenolic compounds a precipitate is formed which interferes with the absorbance. If the measurements were taken at 30 min this difficulty did not arise. Our experience shows that if the absorbance is measured after 30 min, it is possible to obtain consistent and reproducible results. Nevertheless, if one is dealing with relatively small quantities of phenolic compounds, it is possible to use a longer period, e.g., 60 min. The absorbance of the sample tube read against the reagent blank was found to have a distinct pH optimum at 7.5. It was found that the reaction product formed at pH 10 to 11 was stable for 5 to 6 hr after 30 min. Although it was tempting to choose this pH for the final reading in view of this finding, the disadvantage of using this particular condition is that the absorbance of the reagent blank is much higher at this pH than at pH 7.5.

A comparison of this test with three other methods (Thormählen test, ferric chloride test, and ferrocitrate test) for determination of melanogens showed that, in our studies to date, the diazo test is the most sensitive one in detecting melanoma. Moreover, percentage of positive results with this test for patients with other diseases was at least as low as the three other methods (unpublished results). Studies are in progress in assessing the usefulness of this test in following the course of the tumor as well as determining the effects of therapy.

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